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IN VITRO DIAGNOSTIC PRODUCTS FOR HUMAN USE

Proposed Establishment of Product Class Standard for Detection or Measurement of Glucose

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[21 CFR Part 328]

IN VITRO DIAGNOSTIC PRODUCTS FOR HUMAN USE

Proposed Establishment of Product Class Standard for Detection or Measurement of Glucose

In the FEDERAL REGISTER of May 23, 1973 (38 FR 13573), in accordance with procedures established for the development of product class standards for in vitro diagnostic products (§ 167.3, redesignated as § 328.30 (21 CFR 328.30) pursuant to recodification March 29, 1974 (39 FR 11680)), the Commissioner of Food and Drugs published a notice requesting data and information to establish a product class standard for the detection or measurement of glucose or total sugars. The notice described the basis on which the Commissioner was considering the need for such a product class standard and provided an opportunity to all interested persons to comment on the need for such a standard. The Commissioner's concern was based on reported deficiencies in product performance, the serious consequences of inaccurate glucose measurements and the rapid growth of products which were of questionable or unproven reliability.

In response to the notice, a total of 39 submissions of data or information covering 51 marketed products was received. The submissions came from 23 organizations marketing products for the detection or measurement of glucose and from one individual. The products described covered both qualitative, semiquantitative and quantitative products and were based on most of the currently used methodologies for the detection or measurement of glucose.

All such submissions will be made available to the public at the office of the Hearing Clerk, Food and Drug Administration, Rm. 6–86, 5600 Fishers Lane, Rockville, MD 20852, July 29, 1974, except for information which, as provided in § 328.4 (21 CFR 328.4) of the regulations, is demonstrated to constitute a trade secret.

No comments were received regarding the Commissioner's initial determination of the need for a standard, the lack of any adverse effect of a standard upon the utility, cost, or availability of such products, and the lack of any disruption of supply or manufacturing and commercial practices.

Therefore, the Commissioner concludes that the development of this product class standard will have no adverse effect on any of these factors.

The information received revealed basic deficiencies in individual products and problems in those products which indicate the need for a uniform basis for evaluating such products as well as for describing performance.

For example, while several products claim identical or similar performance, the development of those claims was based on very limited data or on nonuniform or inappropriate methods of evaluation. The deficiencies observed include the selection of inappropriate basic methodologies, reference materials and evaluation protocols. In general, performance claims for accuracy, precision and stability were not supported by data from adequately designed studies. Most of the submissions provided inadequate supporting data for stability claims and, generally, lacked labeling information which would allow the user to determine the suitability of the product for use.

The submissions reflected an inconsistent interpretation of the identification and significance of the effect of interfering substances, even among products based on the same methodology.

The Food and Drug Administration, with the cooperation and assistance of its Diagnostic Products Advisory Committee, and the Center for Disease Control (CDC), has reviewed and considered the information submitted.

The Commissioner concludes that (1) the performance and use information provided in the labeling of many of the products is not adequate to provide to the user the information necessary to judge the usefulness of these products or to allow reliance on the results obtained by use of the products, and (2) the information and data submitted in support of many of the products is not adequate to support the product performance claims. Many of the inadequacies relating to instructions for use and interpretation of results will be corrected by labeling revisions proposed for some of these products. Such labeling revisions will not solve the basic problem of the deficiency in the quality of data which must support product performance claims and therefore the actual description of product performance.

The Commissioner recognizes that the measurement of glucose in specimens taken from the human body may be accomplished in a number of acceptable ways. The Commissioner concludes that all such products must provide the same kind and quality of information to the user, i.e., a reliable estimate of the amount of glucose in the specimen.

Therefore, the Commissioner proposes to establish a product class standard for products intended for the measurement or detection of glucose.

Two subclasses of products are identified for purposes of the development of this proposed product class standard. Those products intended to provide an estimate of the exact concentration of glucose in the specimen are included in § 328.100(a), *Quantitative glucose determinations.* Those products intended for the estimation of glucose by less exact means, either because of the manner of final measurement or because of the reaction process used, are included in § 328.100(b), *Qualitative glucose determinations.*

The requirements for accuracy and precision for the qualitative products are less exacting than those for the quantitative products. The manufacturer of a qualitative product, in addition to meeting the minimal requirements, may make additional claims for the product as long as such claims are adequately supported by valid data. Such factors as methodology to be used, composition of reagents, or types and quality of instruments, are not specified in this standard. It is sufficient that this information be provided to the user in the labeling of the product as required in § 328.10(a) and (b) (21 CFR 328.10(a) and (b)).

The product class standard requires that any such products, to be legally marketed, must demonstrate certain qualities of accuracy and precision as described in the standard. Clinical usefulness was the guiding principle in the establishment of the limits of bias, precision and interferences (performance standard parameters). Two kinds of variation were considered: individual biological variation and variation among groups (e.g., geographic distribution, bedridden v. ambulatory). In addition to the data and information submitted in response to the request for information and comment of May 23, 1973, the recommendations of CDC, the Diagnostic Products Advisory Committee and the following investigators in this area have been considered in the establishment of the performance limits incorporated into this standard:

1. Barnett, Roy N., M.D., "Reagent Kits," in Progress in Clinical Pathology, Vol. 4, Ch. 4, pp. 181–198, Grune and Stratton, New York, 1972.

2. Barnett, Roy N., M.D., and Ann D. Cash, MT (ASCP), "Performance of 'Kits' Used for Clinical Chemical Analysis of Glucose," *The American Journal of Clinical Pathology*, 52, No. 4:457–465, 1969.

3. Barnett, Roy N., M.D., "A Scheme for the Comparison of Quantative Methods," *The American Journal of Clinical Pathology*, 43, No. 6:562–560, 1965.

4. Barnett, Roy N., M.D., "Medical Significance of Laboratory Results," *The American Journal of Clinical Pathology*, 50, No. 6:671–676, 1968.

5. Cotlove, Ernest, Eugene K. Harris, and George A. Williams, "Biological and Analytic Components of Variation in Long-Term Studies of Serum Constituents in Normal Subjects, III. Physiological and Medical Implications", *Clinical Chemistry*, 16, No. 12: 1028–1032, 1970.

6. Harris, Eugene K., Paul Kanofsky, George Shakarji, and Ernest Cotlove, "Biological and Analytic Components of Variation in Long-Term Studies of Serum Constituents in Normal Subjects, II. Estimating Biological Components of Variation," *Clinical Chemistry*, 16, No. 12:1022–1027, 1970.

7. Harris, Eugene K., and David L. DeMets, "Biological and Analytic Components of Variation in Long-Term Studies of Serum Constituents in Normal Subjects, V. Estimated Biological Variations in Ionized Calcium," *Clinical Chemistry*, 17, No. 10:983–987, 1971.

8. Wiliams, George Z., D.S. Young, Mervin R. Stein, and Ernest Cotlove, "Biological and Analytic Components of Variation in Long-Term Studies of Serum Constituents in Normal Subjects, I. Objectives, Subject Selection, Laboratory Procedures, and Estimation of Analytic Deviation," *Clinical Chemistry*, 16, No. 12:1016–1021, 1970.

9. Young, D. S., Eugene K. Harris, and Ernest Cotlove, "Biological and Analytic Components of Variation in Long-Term Studies of Serum Constituents in Normal Subjects, IV. Results of a Study Designed to Eliminate Long-Term Analytic Deviations," *Clinical Chemistry*, 17, No. 5:403–410, 1971.

Specifications of accuracy and precision are intended to give the user assurance of the quality of results he may obtain on patient specimens, using the product at any time during its specified useful life. For the purpose of this standard, the term "accuracy" corresponds to the meaning of the term "bias" or "systematic error." "Bias" is the mean difference (positive or negative) between values obtained with the product and values established by the reference method, and is expressed in concentration units or as a percentage (relative bias). Another measure of performance quality is specified, namely "precision," and refers to the degree of agreement of repeated measurements of the glucose content of the same material. A third factor in performance, "specificity,"[2] is partly provided by the requirement for a specified degree of accuracy, and partly by the general labeling requirements for a statement concerning interfering substances.

There is no generally recognized, established reference method available for the measurement of glucose. There are several methods considered to be of high reliability. Three methodologies are proposed as reference methods, each of which has certain advantages and disadvantages. Two of these (ortho-toluidine and glucose oxidase) have the advantage of having been used by laboratory scientists for a substantial period of time and therefore have reasonably well known characteristics. A third method (hexokinase) is currently being studied for its suitability as a reference method at the CDC through a joint effort with the American Association of Clinical Chemists. Because evaluation, field testing, and review of the suitability of these as reference methodologies have not been completed, this proposal contains procedures based on these three methodologies.

Within the limits of their precision capability they are consistent and demonstrate no between-method bias, although occasional discrepancies may be observed in a few pathologic specimens. All three methods employ deproteinized filtrates, partly to minimize sporadic interference in some specimens and partly to abolish differences between methods arising from the slight protein-volume effect in these dilute filtrates.

At some time in the future, possibly by the time of publication of the final order of this proposed product class standard, one or more of these methods may be designated as the sole reference method for this determination.

A detailed protocol for the evaluation of product performance is currently being developed. The protocol will include such things as detailed guidelines for (1) testing procedures, (2) statistical evaluation of results, and (3) criteria for decisionmaking. This document will be made available as soon as possible, but no later than at the time of publication of a final order of this product class standard.

Any expiration date or other representation of product stability is a representation that the product meets the requirements of this product class standard at any time during the period represented by the manufacturer. Failure of the product labeling to bear an expiration date or other representation of product stability is a violation of the requirement in § 328.10(a)(6) that such information must appear on the product label.

The components of any system for measuring glucose in specimens taken from the human body may be broadly described as (1) calibrator, (2) reagents, (3) processing equipment, (4) detector instrument, and (5) analyst. No attempt has been made to identify the relative contributions of these components to the total error in the result obtained. A manufacturer providing a total system must assure that the error contributed by each component which he supplies is controlled so that the total system meets the requirements of this standard. Manufacturers providing only parts of a system must assure that the components they provide, when used with other designated components, and when used as directed in the product labeling, will yield results which meet the requirements of the standard.

Evaluations of products to assure that they meet a performance standard involves the development of sampling protocols, statistical design and judgments and laboratory quality control. The manufacturer must have data to assure that the methodology on which the product is based is suitable. He must then develop and use adequate manufacturing quality control procedures to assure that errors contributed by individual components are identified and controlled so that the product performance meets the requirements of the product class standard.

It is proposed by the Commissioner that all glucose determination products that are subject to the product class standard set forth below and that are initially introduced into interstate commerce beginning one year following publication of the final order in the FEDERAL REGISTER in response to this proposal shall meet such product class standard requirements.

Therefore, pursuant to the provisions of the Federal Food, Drug, and Cosmetic Act (secs. 201, 501, 502, 505, 508, 510, 701, 52 Stat. 1040–1042 as amended, 1049–1053 as amended, 1055–1056 as amended, 76 Stat. 789, 794 as amended; 21 U.S.C. 321, 351, 352, 355, 358, 360, 371) and the Public Health Service Act (sec. 351, 58 Stat. 702 as amended; 42 U.S.C. 262) and under the authority delegated to him (21 CFR 2.120) the Commissioner proposes that 21 CFR Part 328 be amended by adding a new Subpart E, to consist at this time of the following:

Subpart E—Product Class Standards

GLUCOSE DETERMINATIONS

Sec.

328.100 Quantitative glucose determinations.
328.101 Qualitative glucose determinations.
328.102 Reference methods for glucose determinations.

AUTHORITY: Secs. 201, 501, 502, 505, 508, 510, 701, 52 Stat. 1040–1042 as amended, 1049–1053 as amended, 1055–1056 as amended, 76 Stat. 789, 794 as amended; (21 U.S.C. 321, 351, 352, 355, 358, 360, 371); and sec. 351, 58 Stat. 702 as amended; (42 U.S.C. 262.).

Subpart E—Product Class Standards

GLUCOSE DETERMINATIONS

§ 328.100 Quantitative glucose determinations.

This product class standard establishes criteria for the performance of products intended for use in the measurement of the exact concentration of glucose in specimens taken from the human body. Such products include calibrators, controls, reagents, instruments, systems and components, parts or accessories intended for use with such products.

(a) *Labeling.* The general labeling requirements of § 328.10 (a) and (b) apply to all products included in this section. The following specific labeling requirements also apply as indicated:

(1) The statement required by §§ 328.10(a)(2) and 328.10(b)(2) shall be as follows:

(i) For a system, a statement such as "For use in the quantitative determination of glucose". If the product is limited to use with specified specimen types such as urine, serum, etc., this must be stated, e.g., "For use in the quantitative determination of glucose in urine".

(ii) For a calibrator intended to be used in the calibration of a particular system or method, a statement such as "For the calibration of ABC glucose kit". If the calibrator is not intended for, or is not limited to, use with a particular system or method, a statement such as "For the calibration of quantitative glucose methodology".

(iii) For a control material intended for use as such in the quantitative determination of glucose in a particular specimen type (e.g., urine, serum), a statement such as "For use as a control in the quantitative determination of glucose in urine".

(iv) For a reagent intended for use in the quantitative determination of glucose as part of a particular method or system, a statement such as "Reagent for use in the quantitative determination of glucose by the XYZ system".

(v) For instruments intended for use in the quantitative determination of glucose, a statement such as "For use in the quantitative determination of glucose".

(2) In addition to the statement of interfering substances required by § 328.10(b)(10), a precise statement reflecting the effect or noneffect of the presence of those materials listed in paragraph (c)(2) of this section shall be included. If the effect of any of these materials at concentration levels obtained in normal or pathological conditions, drug therapy, drug toxicity or by addition to collected specimens for preservative or similar purposes will cause the product to fail to meet the requirements for bias listed in paragraph (c)(1) of this section, instructions shall be provided to assist the user in identifying the presence of these materials in such specimens. If the effect can be eliminated by appropriate treat-

ment of the specimen or during the analytical process, instructions shall be provided to do so.

(3) If the effects of known interfering substances cannot be corrected by appropriate treatment of the specimen or during the analytical process, the label and labeling shall describe the product as unsuitable for use with specimens containing such substances pursuant to §§ 328.10(a)(2) and 328.10(b)(10).

(4) The statement required by § 328.10(b)(12) shall include a positive statement that the product meets or exceeds the requirements of this standard.

(b) *Calibrator requirements.* A material (calibrator) intended for use as the basis of reference for the measurement of glucose concentration in specimens taken from the human body shall meet the following requirements.

(1) It shall be subject to the product class standard for calibrators.

(2) It shall meet one of the following requirements:

(i) Pure D-glucose shall be not less than 99.5 percent D-glucose, or meet the following requirements for D-glucose anhydrous:

(*a*) Specific rotation $[\alpha]_D^{20°}$, not less than +52.5° nor more than +53.0°.

(*b*) Insoluble matter, not more than 0.005 percent.

(*c*) Loss on drying at 105° C, not more than 0.20 percent.

(*d*) Residue after ignition, not more than 0.020 percent.

(*e*) Acidity (as CH_3COOH), not more than 0.015 percent.

(*f*) Chloride (Cl), not more than 0.010 percent.

(*g*) Sulfate and sulfite (as SO_4), nor more than 0.005 percent.

(*h*) Starch. Dissolve one gram in 10 milliliters of water and add 0.05 milliliter 0.1*N* iodine solution. No blue color should appear.

(*i*) Arsenic (As), not more than 0.00004 percent.

(*j*) Heavy metals (as Pb), not more than 0.0005 percent.

(*k*) Iron (Fe), not more than 0.0005 percent.

(ii) For pure D-glucose in completely defined solvent (e.g., D-glucose in distilled water plus benzoic acid), the glucose concentration (weight/volume (w/v)) shall be within ±0.2 percent of the labeled concentration.

(iii) For D-glucose in an undefined solvent matrix (e.g., in human or animal serum or urine, or synthetic materials), the glucose concentration shall be within ±1.0 percent of the declared value.

(iv) A calibrator which is not or does not contain glucose (such as color charts or solutions) must provide values traceable to values obtained by the use of a calibrator containing glucose. Any such secondary calibrator shall be assigned a numerical equivalent, which shall be assured to be within 1 percent of the declared value.

(c) *Bias and standard deviation.* (1) The product shall measure the glucose content with a bias and standard deviation of no more than that designated in Table I of this paragraph within the concentration range for the specimen. The marketing of components as suitable for use in any system or method constitutes a representation that the system or method meets these requirements and that the individual components will not cause results which deviate from the requirements of this product class standard.

TABLE I

Concentration of glucose in specimen [1]	Maximum standard deviation [2]	Maximum bias [3]
0 to 150 mg/dl (8.33 mM/l) [4]	5 mg/dl (0.28 mM/l)	±5 mg/dl (0.28 mM/l).
150 mg/dl (8.33 mM/l) to 300 mg/dl (16.65 mM/l).	5 mg/dl (0.28 mM/l)	±10 mg/dl (0.56 m/l).
More than 300 mg/dl (16.65 mM/l)	10 mg/dl (0.56 mM/l)	±20 mg/dl (1.66 mM/l).

[1] As measured by the reference method.
[2] Standard deviation is the square root of the variance. Variance is the mean of squared differences between each single result and the mean of all results.
[3] Bias is the mean difference between values obtained with the product and values established by the reference method. Mean refers to the average that could be obtained if unlimited testing was done.
[4] Millimoles per liter.

(2) The effect of each of the substances listed in Table II of this paragraph shall be determined. Such determinations shall also be made for any other substance or patient condition which has been shown to influence the test results. The effects of such substances shall be declared only when such effects might occur at concentrations likely to be reached in normal or pathologic conditions, drug therapy, drug toxicity or by addition to collected specimens.

TABLE II

Endogenous substances	Exogenous substances	Anticoagulants and preservatives
Fructose	L-dopa	Heparin.
Galactose	Salicylates (derivatives and metabolites).	Ethylenediaminetetraacetic acid (EDTA).
Mannose	Dextran	Fluorides.
Ascorbic acid	Xylose	Thymol.
Uric acid	Radiopaque substances.	Citrate.
Creatinine		Oxalate.
Bilirubin		
Glutathione		
Cysteine		
Hemoglobin		
Dihydroxyphenylalanine (dopa)		
Lipids		

(3) Unknown or unspecified interfering substances in native specimens shall be such that no less than 90 percent of the individual differences between results obtained on the same specimens by use of the product and by use of the reference method fall within:

(i) ±10 mg/dl (0.56 millimoles per liter (mM/l) if the reference value is 0 to 150 mg/dl (0 to 8.33 mM/l).

(ii) ±20 mg/dl (1.12 mM/l) if the reference value is 150 to 300 mg/dl (8.33 to 16.65 mM/l).

(iii) ±40 mg/dl (2.24 mM/l) if the reference value is greater than 300 mg/dl (16.65 mM/l).

(d) *Test procedures and requirements.* The following test procedures and requirements shall apply.

(1) All products shall be tested using the product as it is marketed. Materials not furnished as a part of the product but indicated in the labeling as necessary for proper use of the product shall be used in evaluation of the product. Any such materials which meet the specifications of the manufacturer may be selected. If the labeling indicates that alternative materials are considered acceptable, any such materials may be selected for product testing purposes. Testing for compliance may include testing with any specimen type claimed. Any product which fails to conform to the performance requirements established in paragraph (c) of this section is not in compliance with this standard.

(2) Testing for compliance may be done at any time during the life expectancy of the product. The product shall be handled as stated in the labeling instructions for storage, reconstitution, mixing and stability; e.g., if the labeling states that a reagent product stored at room temperature is stable for one year, and after reconstitution and dilution is stable for one month when stored in a refrigerator, testing for compliance shall be done in accordance with the stated conditions and within that period of time; and if the manufacturer indicates a need for instrument calibration once a year, no calibration will be done at less than that interval for the purpose of product evaluation. An expiration date or representation of the period of product stability constitutes a representation that the product meets the requirements of the standard at any time during the period represented. If there is no expiration date or representation of the period of product stability the product will be deemed to be in violation of the requirement in § 328.10(a)(6) that such information appear on the product label.

(3) To evaluate the precision and bias of results which may be obtained by use of a product, specimens shall usually be selected which have glucose concentrations at or near the midpoint of those concentration ranges listed in paragraph (c)(1) of this section, except that the maximum concentration shall not exceed the level which is represented as

the maximum level of usefulness for the product. Specimens used for product evaluation shall be of the same matrix for which the product is represented as useful. Such specimens shall be stable with regard to glucose content throughout the evaluation period. Specimens used in product evaluation will consist of:

(i) Calibrators supplied or specified by the manufacturer.

(ii) A control material supplied or specified by the manufacturer.

(iii) Pure solutions with glucose content defined (weight/volume basis).

(iv) Samples from stable pools of human or animal origin simulating patient specimens.

(v) Native specimens from a hospital or clinical population, including specimens from patients with diabetes or diabetes-associated disease.

(vi) Samples from a serum or other appropriate matrix pool, to which have been added known amounts of suspected interfering substances.

(4) Any test scheme shall be adequate to assure the user that those variables which have been specified as requiring control are included in the assessment. No special effort shall be made to control any other laboratory conditions which might affect the performance of the products. Ordinarily any test scheme shall include in the assessment of precision and bias any lot-to-lot and within-lot variation in the individual components of the test, any changes in the product due to aging, and short-term (day-to-day) and long-term (week-to-week) variations in the testing laboratory. The testing scheme shall be such that the probability of declaring the standard has been met, when in fact unlimited testing would show that the standard is not met, shall not exceed 5 percent when:

(i) The bias exceeds the standard by 10 percent;

(ii) The precision exceeds the standard by 10 percent;

(iii) The percentage of individual differences between results obtained on native specimens by the product and the reference method with the limits given in paragraph (a)(3) of this section is 5 percentage points less than the performance requirement.

§ 328.101 Qualitative glucose determinations.

This product class standard establishes criteria for the performance of products intended for use in the detection of glucose in specimens taken from the human body. Such products include calibrators, controls, reagents, instruments, and components, parts, or accessories intended for use with such products. In addition to detecting glucose, such products may also provide procedures for estimating the amount of glucose present. The precision or specificity obtained by use of such products is less than that ordinarily obtained by use of those products subject to the requirements of § 328.100 governing quantitative glucose determinations.

(a) *Labeling.* The general labeling requirements of §§ 328.10 (a) and (b) apply to all products included in this section. The following specific labeling requirements also apply as indicated.

(1) The statement required by §§ 328.10(a)(2) and 328.10(b)(2) shall be as follows:

(i) For a system, a statement such as "For use in the qualitative detection of glucose". If the product is limited to use with specific specimen types such as urine, spinal fluid or blood, a statement such as, "For use in the qualitative detection of glucose in blood".

(ii) For a calibrator intended for use in the calibration of a particular system or method, a statement such as "For the calibration of DEF Glucose Kit". If the calibrator is not intended for, or limited to use with a particular system or method, a statement such as "For the calibration of qualitative glucose methodology".

(iii) For a control material intended for use as a control in the qualitative detection of glucose in a particular specimen type, e.g., urine or serum, a statement such as "For use as a control in the qualitative detection of glucose in urine".

(iv) For a reagent intended for use in the qualitative detection of glucose as part of a particular method or system, a statement such as "Reagent for use in the qualitative detection of glucose by the EFG system".

(v) For instruments intended for use in the qualitative detection of glucose, a statement such as "For use in the qualitative detection of glucose".

(2) In addition to the statement of interfering substances required by § 328.10(b)(10), a precise statement reflecting the effect or noneffect of the presence of those materials listed in paragraph (c)(2) of this section shall be included. If the effect of any of these materials at concentration levels obtained in normal or pathological conditions, drug therapy, drug toxicity or by addition to collected specimens for preservative or similar purposes will cause the product to fail to meet the requirements of this standard, instructions shall be provided to assist the user in identifying the presence of these materials in such specimens. If the effect can be eliminated by appropriate treatment of the specimen or during the analytical process, instructions shall be provided to do so.

(3) If the effects of known interfering substances cannot be corrected by appropriate treatment of the specimen or during the analytical process, the label and labeling shall describe the product as unsuitable for use with specimens containing such substances pursuant to §§ 328.10(a)(2) and 328.10(b)(10).

(4) The statement required by § 328.10(b)(12) shall include a positive statement that the product meets or exceeds the requirements of this standard.

(b) A material intended for use as the basis for assignment of qualitative glucose values to specimens shall meet the following requirements:

(1) It shall be subject to the product class standard for calibrators.

(2) It shall meet one of the following requirements.

(i) If it contains glucose, it shall meet the requirements for calibrators in § 328.100(b) except that the assurance of concentration of glucose in an undefined matrix shall be ±10 percent of the declared value.

(ii) If it is not or does not contain glucose, it must provide values traceable to values originally obtained by the use of a calibrator containing glucose. Any such secondary calibrator (such as a color chart or solution) shall provide a numerical equivalent of the range of values for which it is offered for use. The midpoint value of each of these ranges shall be assured within ±10 percent of the glucose concentration.

(c) *Detection range; effect of other substances.* (1) The product shall detect glucose within the limits specified in Table I of this section for urine and blood. The minimum number of ranges of detection necessary to provide useful information are as indicated in Table I of this section. The marketing of components as suitable for use in any system or method constitutes a representation that the system or method meets these requirements and that the individual components will not cause results which deviate from the requirements of this product class standard. If the product labeling makes representations of usefulness at or within glucose concentration ranges other than those listed in Table I of this section, each range and its midpoint shall be stated and not more than 10 percent of specimens having a glucose concentration at that midpoint shall give results outside the stated range.

TABLE I

Concentration of glucose [1]	Results must be—	Expected result—	Remarks [2]
Urine:			
0 to 20 mg/dl (0 to 1.11 mM/l)	Negative		
Greater than 250 mg/dl (13.86 mM/l).	Positive		
Blood (whole blood, serum or plasma):			
0 to 20 mg/dl (0 to 1.11 mM/l)		Abnormal low	Specimens in this range shall provide an "abnormal low" result.
20 to 60 mg/dl (1.11 to 3.33 mM/l)		do	A specimen at 40 mg/dl (2.22 mM/l) should not be declared "normal" more than 10 percent of the time, and shall never be declared "abnormal high."
60 to 120 mg/dl (3.33 to 6.66 mM/l)		Normal	A specimen at 90 mg/dl (5.00 mM/l) shall not be declared "abnormal" more than 10 percent of the time.
120 to 200 mg/dl (6.66 to 11.1 mM/l)		Abnormal high	A specimen at 160 mg/dl (8.88 mM/l) shall not be declared "normal" more than 10 percent of the time, and shall never be declared "abnormal low."
Greater than 200 mg/dl (11.1 mM/l)		do	A specimen in this range shall provide an "abnormal high" result.

[1] As measured by the reference method.
[2] The results obtained by use of a product may not indicate glucose concentrations beyond the next contiguous range.

(2) The effect of each of the substances listed in Table II of this section shall be determined. Such determinations shall also be made for any other substance or patient condition which has been shown to influence the test results These effects shall be declared only when they might occur at concentration likely to be reached in normal or pathologic conditions, drug therapy, drug toxicity or by addition to collected specimens.

TABLE II

Endogenous substances	Exogenous substances	Anticoagulants and preservatives
Fructose	Dextran	Thymol.
Galactose	Salicylates (derivatives and metabolites).	Fluorides.
Mannose	Hydrogen peroxide	
Pentoses	Hypochlorites	
Ascorbic acid		
Uric acid		
Creatinine		
Bilirubin		
Dihydroxyphenylalanine (dopa)		
Glutathione		
Hemoglobin		
Lipids		

(d) *Test procedures and requirements.* The following test procedures and requirements shall apply:

(1) All products shall be tested using the product as it is marketed. Materials not furnished as a part of the product but indicated in the labeling as necessary for proper use of the product shall be used in evaluation of the product. Any such materials may be selected that meet the specifications of the manufacturer. If the labeling indicates alternative materials are considered acceptable, any such materials may be selected for product testing purposes. Testing for compliance may include testing with any specimen type claimed. Any product which fails to conform to the performance requirements established in paragraph (c) (1) of this section is not in compliance with this standard.

(2) Testing for compliance may be done at any time during the life expectancy of the product. The product shall be handled as stated in the labeling instructions for storage, reconstitution, mixing and stability; e.g., if the labeling states that a reagent product stored at room temperature is stable 1 year, and after reconstitution and dilution is stable for 1 month when stored in a refrigerator, testing for compliance shall be done in accordance with the stated conditions and within that period of time; and if the manufacturer indicates a need for instrument calibration once a year, no calibration will be done at less than that interval for the purpose of product evaluation. An expiration date or representation of the period of product stability constitutes a representation that the product meets the requirements of the standard at any time during the period represented. If there is no expiration date or representation of the period of product stability the product will be deemed to be in violation of the requirement in § 328.10(a)(6) that such information appear on the product label.

(3) A product intended for use in the detection of glucose in urine shall be tested using specimens with glucose concentrations below 20 mg/dl (1.11 mM/1) and above 250 mg/dl (13.86 mM/1). Any product intended for use in the detection of glucose in blood shall be tested using specimens with glucose concentrations as indicated in paragraph (c)(1) of this section.

(4) Any testing scheme shall be adequate to assure the user that those variables which have been specified as requiring control are included in the assessment. No special effort shall be made to control any other laboratory conditions which might affect the performance of the products. Ordinarily any test scheme shall include in the assessment of precision and bias any lot-to-lot and within-lot variation in the individual components of the test, any changes in the product due to aging, and short-term (day-to-day) and long-term (week-to-week) variations in the testing laboratory, as well as those variations to be expected in the reading and interpretation of results such as color comparisons. The testing scheme shall be such that the probability of declaring that the product has met the performance requirements is less than 5 percent when unlimited testing would show that any one of the following is true.

(i) The percentage of specimens with glucose concentrations of approximately 40 mg/dl (2.22 mM/1) which will be declared "normal" is greater than 15 percent.

(ii) The percentage of specimens with glucose concentrations of approximately 90 mg/dl (5.00 mM/1) which will be declared "abnormal low" or "abnormal high" is greater than 15 percent.

(iii) The percentage of specimens with glucose concentrations of approximately 160 mg/dl (8.88 mM/1) which will be declared "normal" is greater than 15 percent.

(5) A minimum of 200 specimens should be examined in each concentration range. However, in those performance categories requiring absolute reliability (0 percent or 100 percent), the product will be considered as not meeting the performance requirements upon the first result which deviates from the requirement.

§ 328.102 Reference methods for glucose determination.

In order to assess bias, a means of establishing the concentration of glucose in specimens is required. For the purpose of this product class standard, one of the following reference methods shall be used. The sections on sample handling, preservation and storage, general procedural approaches, material specifications, and data evaluations and computations are common to each of the described methods.

(a) *Sample handling, preservation and storage.* (1) The primary precaution in the handling of any glucose solution is the prevention of glycolysis. In the cell-free samples for which this method is designed, the preponderant source of sample deterioration is microbiologic, and preservation of original glucose concentration requires avoidance of bacterial contamination. Evaporation from small samples can be a serious problem as can chemical contamination in minute quantities. Therefore, sample vessel cleanliness, sterility and tight closure are rigorous requirements for proper handling and storage.

(2) Glucose standards are dissolved and diluted in 1 gram/liter benzoic acid solutions, which gives secure long-term protection against microbiologic contamination at 4° C and adequate protection for some weeks at room temperature. Aqueous dilutions of these standards or other dilute solutions of glucose, and re-

constituted lyophilisates are extremely labile to glycolysis without aseptic handling or the addition of bacteriostatic agents.

(3) The biologic specimens to be used as samples for this analysis are limited to serum, plasma, spinal fluid or urine, all of which present hazards of cellular as well as bacteriologic glycolysis and require special precautions in preparation and storage. Lyophilized serum products are not necessarily free of these contaminants, and often show greater lability to glucose degradation than freshly collected native serum. Collection of blood and spinal fluid is ordinarily performed under aseptic conditions; urine is rarely so collected. Even if preservatives are used, pools of any of these materials that are to be processed as control or calibrator sample surrogates must be membrane-filtered to remove both tissue cells and microorganisms, since there are few complete inhibitors of cellular glycolysis and many such substances present interference problems. Therefore, plasma or serum must be centrifuged to produce essentially cell-free supernatants for materials that are to be stored for any period of time, and an adequate preservative should be added either with the anticoagulant or to the harvested serum. The anticoagulants heparin, oxalate, citrate and ethylenediaminetetraacetate (EDTA) do not interfere with the analysis. Sodium fluoride (NaF) is a secure preservative, but as the sole anticoagulant the high concentration necessary produces unsuitable plasma and may interfere with the analysis. Anticoagulants other than heparin cause osmotic shifts of cellular water and other effects in blood specimens that produce alterations in plasma glucose concentration.

(4) NaF at a concentration of 2.5 milligrams per milliliter of blood is the recommended preservative. The NaF is best combined with 1 milligram per milliliter of disodium EDTA. This mixture is availableinconventionalvacuu m collection tubes.

(5) Immediate and thorough mixing of the freshly collected specimen is essential to dissolve the dry anticoagulant to prevent later problems with fibrin in frozen plasma.

(6) The keys to adequate storage of specimens are sterility, secure closure, low temperature and freedom from cellular or chemical contamination. For storage longer than 48 hours, even preserved specimens should be frozen in containers that are securely closed and have a low permeability to water vapor. Long term storage for many plastic vials may require sealing in cans with saturated blotting paper; heavy glass vials are preferred. Unless a good seal is obtained by special compression fittings, screw caps may not be reliable. Thawing of frozen specimens should be performed rapidly, i.e., in a water bath at a temperature no higher than 30° C. Repetitive sampling from the same vial presents the risk of potential deterioration of the specimen from repeated thawing, evaporation and contamination.

(b) *General procedural approaches.* (1) Any reference method must aim at high capability for accuracy and precision and must be defined to the extent possible. Accuracy capability can rarely be described in absolute terms, since it results from the conditions and procedures used to determine the scientific or analytic method. Estimates of bias may be made for recognized interfering substances, but the magnitude of bias in any one result is determined by the nonspecificity of the method combined with the interfering components in each specimen. The use of a reference method assumes acceptance of an arbitrary accuracy capability.

(2) The precision component of any final result represents the sum of the variations which result from:

(i) The volumetric manipulations on the sample;

(ii) The ease or difficulty of reproducing prescribed conditions; and

(iii) The response-measuring system.

The procedures and specifications prescribed in these methods of reference are considered essential to achieving their precision capabilities in a practical operation.

(3) (i) Defining volumes are liquid or solution volumes that define concentration volumetrically, i.e., mass or activity or molecular number per unit volume. These comprise (*a*) "primary standard solutions" prepared by dissolving an accurately known mass of defined substance in an accurately known volume of total solution; (*b*) "primary" or "original specimens" of solutions to be analyzed on a volumetric basis; and (*c*) "volumetric dilutions" of either standards or specimens that define an aliquot by means of a precisely known dilution factor.

(ii) Liquid aliquot is an exactly known volume of solution, delivered as a proportional part of an original specimen or standard solution.

(iii) Aliquant is an inexactly defined proportional sample of a parent volume, the proportionality of which is not necessarily known or used in relating the concentration of original sample to its final dilution. Since it may be delivered with great repetitive precision, an inexactly known volume of an aliquant may be used in a comparison technique in precise quantitative determinations of concentration, referred to some calibrating or standard solution.

(iv) Comparison technique: In the procedures set forth in paragraph (e) of this section, the two steps (deproteinization and final color reaction) are conducted by identical treatment of aliquants of specimens and working standard solutions. The reaction response of the specimen is then quantified by referring it to that of a precisely defined and identically treated standard solution. Although these steps must be carried out with high precision, the volumes of aliquants of samples and diluents or reagents need not be exactly known, and primary calibration of the volumetric measuring system is not essential as long as the aliquants are precisely reproduced.

(c) *Material specifications*—(1) *Volumetric measurement*—(i) *Precision tolerance.* The system for delivering any liquid volume in the analysis should have a precision capability of the measurement for repetitive deliveries within ±0.2 percent of the target mean; the precision of the volumes delivered depends upon operator care. Ultimate precision capability is achieved by the use of one pipet for the delivery of all samples and standard solutions. The use of multiple pipets for the same task requires selection in which this tolerance can be assured. Products of leading manufacturers can usually be relied upon to meet these tolerances; however, the user must identify reliable sources and verify precision.

(ii) *Accuracy tolerance.* For all defining volumetric measurements, such as dilutions of standard solutions or of specimens prior to the analysis, pipets and volumetric flasks conforming to National Bureau of Standards (NBS) tolerance, Class A, Fed. Spec. NNN–F–00289a (May 21, 1965) and NNN–P–00395a (May 27, 1965)[1] must be used. The accuracy capability of the measurement in these applications should fall within ±0.2 percent of the target volume. Class A pipets meet this requirement only at volumes above 2 milliliters. The mean volume of any reagent or sample delivered in a comparison technique must be within ±5 percent of the volume specified in the procedure.

(iii) *Sample deliveries.* Since the technique of comparison with concomitant, identically processed standard solutions is employed here, only the precision tolerance is critical. In measurements of original sample aliquants (specimens and working standards) the effect of differing viscosities, e.g., between serum and aqueous standards, on the volume delivered must be eliminated. Acceptable alternative methods are: (*a*) A single transfer or Ostwald pipet is used for all deliveries of sample or deproteinized supernate. Regardless of its calibration mode, it is used as "to contain" (TC) pipet, with a flushing of any residual material by two complete fillings with the recipient water or reagent, and positive expulsion. Between individual samples the pipet must be well drained, wiped and given three thorough rinses with the next sample to be delivered.

(*b*) A set of pipets may be used which has been verified as falling within a tolerance of ±0.2 percent of some contained volume that lies within ±5 percent of the volume specified in the procedure. A new pipet is used for each sample with flushing of the material with the recipient reagent. The only commercially available pipets with tolerances within 0.2 percent in the 1-milliliter size are 1000 microliter micropipets, e.g., Kirk or Lambda types, conforming to American

[1] Copies may be obtained from: National Bureau of Standards, Department of Commerce, 14th, E, and Constitution Ave. NW., Washington, D.C. 20230.

Chemical Society (ACS) specifications.[3]

(c) Certain syringe-type pipets with precisely limited stops may be available with the necessary precision of repetitive delivery if they represent all-liquid devices (not air-pistol pipets) in which the sample is measured by positive displacement, rather than by containment. In this case, the pipet is not flushed with reagent, but must be rinsed three times with the next sample.

(d) Several precision sample-diluting devices that measure the sample by aspiration in an all-liquid system and deliver it by positive displacement and flush out with water or reagent can qualify. The actual volume delivered must be within ±5 percent of the specified sample volume and the device must be demonstrated to achieve repetitive deliveries with a precision of 0.2 percent [1 coefficient of variation (C.V.)]. The sample and reagent contacting surfaces of such a device must be demonstrated not to be structurally deformable or reactive with any reagent or sample.

(2) *Photometric measurement*—(i) *Spectral resolution and wavelength.* A half bandwidth of 10 nanometers centered within ±5 nanometers of the specified wavelength for measurements at 540 and 630 nanometers; measurements at 340 nanometers must be centered within ±2 nanometers, with a resolution of 2 nanometers.

(ii) *Photometric linearity.* (a) Within 0.001 absorbance from 0.000 absorbance to 0.500 absorbance.

(b) Within 0.002 absorbance from 0.500 absorbance to 1.000 absorbance.

(c) Within 0.003 absorbance from 1.000 absorbance to 1.500 absorbance.

(iii) *Photometric precision, including noise, at 340 nanometers.*

(a) Within 0.001 absorbance at 0.000 absorbance to 0.100 absorbance.

(b) Within 0.003 absorbance at 0.500 absorbance.

(c) Within 0.005 absorbance at 1.000 absorbance.

(iv) *Stability, less noise, at 340 nanometers.* (a) Zero drift not greater than 0.001 absorbance per hour.

(b) Drift at 1.000 absorbance not greater than 0.003 absorbance at the required aperture and energy level.

(v) *Stray light.* For measurements at 340 nanometers, stray light must be less than 0.2 percent.

(vi) *Cuvet system.* Cuvets with plane-parallel faces with a pathlength of 10 millimeters are used. A 10 millimeter flow-through cuvet is acceptable if it can be adequately flushed to eliminate all carryover of sample and if its pathlength has been verified.

(3) *Glassware.* (i) All glassware shall be chemically clean, including at least two final rinses with demineralized, distilled water, and dried in a dust-free environment.

(ii) If individual cuvets are used for photometric measurements, they shall be matched to a sufficiently close tolerance to satisfy the precision requirement given under paragraph (c)(2) of this section, or corrections for differences in pathlength must individually be applied. Normally, a common cuvet, thoroughly rinsed between readings with the next sample, or a flow-through cuvet, is used. These shall be cleaned after each analytical run.

(iii) Except where plastic dispensing bottles are specified, all reagents and stock standards are to be stored in borosilicate glass vessels. Tight-fitting caps must be fitted with either flat Teflon liners or polyethylene conical closures.

(iv) Tubes for the preparation of deproteinized supernates must contain a volume of at least 23 milliliters and must be capable of being securely capped with nonreactive closures. Provisions must be made either for centrifuging the tubes directly or for transferring smaller volumes of the mixture to smaller tubes.

(4) *Chemicals*—(i) Reagent water: Demineralized feed water, run through an active charcoal and a high efficiency mixed-bed demineralizer column (effluent resistivity of at least 1 megohm) is distilled in an all-grass vented still operated at two-thirds of the rated output. It is collected and stored in borosilicate glass containers.

(ii) Carbon dioxide (CO_2)-free water: Reagent water boiled for 15 minutes in an open flask and allowed to cool covered with a watch glass. This is prepared at the time of use.

(iii) Glucose standard: Standard glucose solutions are prepared from National Bureau of Standards certified D-glucose, Standard Reference Material No. 917,[3] which has been stored in the laboratory in its original capped bottle in a vacuum desiccator over anhydrous calcium sulfate.

(iv) Chemicals designated as "ACS grade" must meet ACS analytical reagent specifications ("Reagent Chemicals," ACS, 4th ed.[3]).

(5) *Preparation of primary standard solution*—(i) *Benzoic acid diluent, 1.0 gram/liter.* 2.0 grams of ACS-grade benzoic acid are dissolved in 2000 milliliters of hot reagent water in a large flask. Stir thoroughly into solution, and cover while it cools to room temperature. Decant from any fine residue into reagent bottles. Store at room temperature, tightly stoppered.

(ii) *Glucose stock standard, 10.00 grams/liter.* Weigh and transfer 5.000 ±0.002 grams of anhydrous NBS D-glucose into a Class A 500 milliliter volumetric flask; benzoic acid diluent is used to wash in and dilute the glucose until the flask is about two-thirds full. Mix the glucose into complete solution with swirling. Add benzoic acid solution to a point about 1 centimeter below the 500-milliliter mark, and place the flask in a water bath at 20°±1° C, covering the flask just to the neck. Stopper, and let the solution equilibrate for 30 minutes before making to exactly 500-milliliter volume with the benzoic acid diluent. Stopper and mix well by inverting the flask at least 10 times, swirling the inverted flask for 10 seconds at each inversion. Transfer 80 to 85 milliliters to each of 6 screw-cap 4-ounce borosilicate bottles. The labeled and dated bottles are tightly capped and stored at 4° C. One bottle is used for the preparation of a complete set of standards; other convenient volumes may be used for preparing standards having a glucose concentration above 250 mg/dl (13.88 mM/1). Although this stock standard is stable for long periods of time if adequately prepared and handled, it is recommended that new stock standard be prepared each 6 months. Before bottling a new lot, prepare a new 200 mg/dl (11.10 mM/1) working standard and verify its integrity by comparison in replicate in a run with the old set of standards.

(iii) *Glucose working standards.* (a) Prepare a complete set of working standard solutions of pure glucose; equilibrate a bottle of stock glucose standard and of benzoic acid diluent to 20°±1° C in a water bath for 30 minutes. Deliver the aliquots of stock standard listed below into 100 milliliter Class A volumetric flasks and dilute almost to volume with the benzoic acid diluent; final adjustment to the mark is made after another 15 minutes of equilibration of the flasks in the bath. A special set of Class A volumetric flasks and 5-, 10- and 25-milliliter volumetric (TD) pipets and volumetric flasks is reserved for preparing standards.

Stock standard (milliliters)	Total volume (with benzoic acid) (milliliters)	Final glucose concentration (milligrams/deciliter)
5.00	100	50(2.78 mM/l)
10.00	100	100 (5.55 mM/l)
15.00	100	150 (8.33 mM/l)
20.00	100	200 (11.10 mM/l)
25.00	100	250 (13.88 mM/l)

If the desired analytical range is not covered by these standards, additional working standards must be prepared: 300, 400 and 500 mg/dl (16.65, 22.2 and 27.8 mM/1, respectively) working standards may be prepared by dilutions of 30-, 40- and 50-milliliter aliquants, respectively, of stock standard into 100-milliliter volumetric flasks. A complete set of standards is prepared at one time and if any one is depleted or suspect, the entire batch is replaced. The old and new sets are compared by duplicate analysis in one analytical run. A 25 mg/dl (1.39 mM/1) standard may be prepared by mixing 5.0-milliliter aliquots each of 50 mg/dl (2.78 mM/1) standard and benzoic acid diluent in a clean, dry tube.

(b) Working standards are stored in 4-ounce screw-capped polyethylene bottles (squeeze dropping type with snap-cap on the tip) at 4° C for up to 1 month. Cleaning of plastic storage bottles is critical, since gross contamination cannot be seen and since molds may be transferred from one solution to another. Although benzoic acid is a good preservative against ordinary glucolytic micro-

[3] Copies may be obtained from: Journal of Analytical Chemistry, American Chemical Society, 1155 16th St., Washington, D.C. 20036.

[1] See footnote 1 at p. 24141, *supra*.

[2] See footnote 2, 1st col.

organisms, it cannot protect against heavy bacterial inoculations.

(c) On each analytical day, the required working standards are dispensed into clean tubes for equilibration to 20° C, and the stock bottles returned to the refrigerator.

(d) *Data evaluation and computations.* The following guidelines for data evaluation and computations are applicable to each of the reference methods in this standard.

(1) *Drift evaluation.* Drift is identified by a significant alteration of either the response to concentration (y/x) slope between the initial and final absorbance readings of the standard curve, or by a vertical displacement of the two curves throughout the concentration range. The following procedure provides a test for identifying drift.

(i) If the initial and final blank differ by more than 0.005 absorbance, discard run.

(ii) Discard any standard for which the duplicate readings differ by more than 5 percent.

(iii) Sum the absorbance readings for the two lowest standards in the initial set of standard readings, normally the 50 (2.78) and 100 mg/dl (5.55 mM/l) standards.

(iv) Similarly, sum the initial readings for the two highest standards, normally 200 (11.10) and 250 mg/dl (13.88 mM/l standards.

(v) Obtain similar sums for the two lowest and two highest in the final set of standards.

(vi) If the sums for the initial and final absorbance values for the two lowest standards differ by more than 3 percent, discard run. For all such comparisons the lower of the two values is used as the denominator. Similarly, if the difference between initial and final sums for the two highest standards is more than 3 percent, discard the run.

(2) *Validation of duplication standards.* (i) Discard any standard for which duplicate absorbances differ by more than 3 percent, or 5 percent for the 50 mg/dl (2.78 mM/l) standard.

(ii) The data for at least four standard concentrations must be acceptable by this test, or the run must be discarded.

(3) *Obtaining corrected readings.* (i) Average the duplicate values for the blanks and each acceptable standard.

(ii) Subtract the average blank absorbance from the average absorbance for each standard and from the absorbance for each specimen to obtain the "corrected reading."

(4) *Obtaining factor for each standard level.* Obtain a factor for each standard by dividing its concentration by its corrected reading.

(5) *Validation of linearity of the standard curve.* (i) Add the factors for the two lowest accepted standards, normally 50 (2.78) and 100 mg/dl (5.55 mM/l).

(ii) Add factors for the two highest accepted standards, normally 200 (11.10) and 250 mg/dl (13.88 mM/l).

(iii) If these two sums obtained differ by more than 2 percent of the lower value, discard run.

(6) *Obtaining multiplier factor (F) and computing concentrations.*

(i) Average the factors for all accepted standards to obtain F.

(ii) Multiply corrected reading for each specimen by F to obtain glucose concentration.

(iii) Samples with readings more than 10 percent higher than the highest standard accepted for the run, or lying beyond the previously demonstrated linear range, are diluted 1:2 (equal volumes) with reagent water and reanalyzed; the result is adjusted for the dilution factor (2).

(7) *Example, using data derived from the ortho-toluidine method.* The following data were obtained by manual techniques, employing a common 1-milliliter Ostwald pipet for all samples and a precise spectrophotometer using a self-filling cuvet system.

Standard concentration (mg/dl)	Initial values (absorbance)	Final values (absorbance)	1 Test of drift (sum of absorbance)	2 Test of duplication (percent)	3 Corrected readings (absorbance)	4 Factors (F)	5 Test of linearity (sum of F)
0	0.007	0.004	0.005				
50	.086	.085	.248	−1.2	0.0800	625	
			vs.				1260
100	.162	.164	.249	+1.2	.1575	635	
			0.4%				
150	.240	.239		0	.2340	640	
200	.321	.315	.717	−2.0	.3125	640	
			vs.				1286
250	396	.338	.703	−2.0	.2865	646	
			2.0%				2.0%

Although there was evidence of fading of color at the end of this long run, the drift was acceptable (0.4 percent for the two low standards and 2.0 percent for the two high standards). Duplication fell within limits for the blanks and all standards (less than 3 percent). All standard values were acceptable. The test for linearity was barely satisfied, with the sum of the two highest standard factors exceeding that of the two lowest by exactly 2.0 percent, most probably due to excessive fading at the higher concentrations. The overall multiplier factor (F) averaged 637.

(e) *Methods for determination of glucose*—(1) *Ortho-toluidine method for determination of glucose in serum or plasma*—(i) *General.* The ortho-toluidine reaction is considered to be relatively specific for aldohexoses. Actually, certain other sugars may participate in the reaction to some extent, and the usefulness of this method in blood sugar determinations depends upon the fact that glucose is the preponderant sugar and, normally, the only significant reactant in serum or plasma. Specimens from some uncontrolled diabetics and those containing certain glycosans which can hydrolyze in the hot acid reaction can yield appreciably higher values than the actual glucose content. The reaction involves the condensation of glucose with a primary aromatic amine in hot acetic acid to produce an equilibrium mixture of a glycosylamine and a Schiff base:

Ortho-toluidine method

$$CH_3C_6H_4NH_2 + C_6H_{12}O_6 \rightarrow \text{green chromogen}$$

The relatively stable green color produced conforms to the Beer-Bouguer Law up to about 400 mg/dl (22.2 mM/l) glucose concentration.

(a) The method is simple and straightforward, requiring only two reagents, the first for protein precipitation and a second for the color reaction. Deproteinization is required for the most reliable results. Interference from pigmented or uncharacterized components in occasional specimens is minimized by deproteinization.

(b) The o-toluidine reagent is compatible with several common protein-free filtrates of serum or plasma. The reaction volumes are readily adjustable, within limits, for the sensitivity and concentration range desired. However, sources of o-toluidine vary, and some variation in the responses between pure aqueous standards and serum or plasma filtrates may occasionally be observed with some reagents. The characteristics and properties of an acceptable reagent have not been completely defined. Technical grade o-toluidine has often been used; the presence of thiourea has been suggested to compensate for "impurities." Verification of the adequacy of each lot of source reagent is necessary. The present procedure is an optimization of the basic method of Dubowski (Dubowski, K. M., *Clinical Chemistry*, 8: 215–235, 1962 [2]).

(ii) *Reagents and chemical specifications for the o-toluidine method*—

(a) *Chemical specifications*—(1) *Trichloroacetic acid (TCA).* ACS specifications (4th ed., p. 617 [3]). (Trichloroacetic acid is very caustic and must be handled with care.)

(2) *Ortho-toluidine.* Technical grade o-toluidine is distilled under vacuum in the laboratory and kept tightly capped in a vacuum desiccator in the dark at room temperature (o-toluidine or its contained impurities is considered to be a potential carcinogen; contact with the liquid or the color reagent should be strictly avoided.)

[2] See footnote 2 at p. 24142, *supra*.

[3] Copies may be obtained from: Clinical Chemistry Business Office 1725 K St. NW., Washington, D.C. 20006.

(3) *Acetic acid, glacial.* ACS specifications (4th ed., p. 34 *). Acetic acid, glacial, may cause burns of the skin and must be handled with care.

(4) *Thiourea.* Reagent grade.

(b) *Reagent specifications—(1) Protein precipitant TCA.* Trichloroacetic acid solution, 60 grams/liter. Quickly weigh 60±0.5 grams of ACS reagent grade trichloroacetic acid (CCl_3COOH) into a 600-milliliter beaker, add reagent water to dissolve completely; transfer and dilute to 1000 milliliters in a volumetric flask. Store at 4° C in a glass-stoppered reagent bottle (well-designed closures of nonreactive plastics are acceptable). This preparation should not be used after 3 months.

(2) *o-Toluidine color reagent.* Completely dissolve 1.5±0.1 grams of reagent grade thiourea in 940±5 milliliters of ACS glacial acetic acid in a beaker. Warm to 50° C and stir constantly in a fume hood. When the thiourea has dissolved, cool in a water bath to room temperature, and add 60±0.5 milliliters of ortho-toluidine. Transfer to a 1-liter amber bottle, mix thoroughly, and store at room temperature for up to 3 months.

(3) *Working standards.* The preparation of primary standard solutions is described in paragraph (c)(5)(iii) of this section.

(iii) *Procedure for ortho-toluidine analysis of glucose—(a) Preparation of deproteinized supernates.* (1) Pour sufficient portions of each standard into clean, cappable tubes and return standards to refrigerator. Bring specimens, standards, and reagent water to constant temperature by placing for 30 minutes in a temperature-controlled water bath at room temperature (15° to 30° C). Label a sufficient number of 18 x 150 millimeter cappable tubes to accommodate:

Type of sample:	*Preparation*
Duplicate blanks (zero concentration standard).	Prepared with benzoic acid diluent as a sample and carried through the procedure as a reagent blank.
Duplicate working standards	50, 100, 150, 200, and 250 mg/dl (2.78, 5.55, 8.33, 11.10 and 13.88 mM/l, respectively) standards. If samples above 275 mg/dl (15.26 mM/l) are to be analyzed, include the appropriate working standard.
Two biologic control samples	Appropriate concentration and analytical history.
Specimens	Specimens above 300 mg/dl (16.65 mM/l) require dilution with water before deproteinization.

(2) Deliver 10.0 milliliters of reagent water into each tube, using the same volumetric pipet. If automatic dilution is employed, this water is used as the fluid to flush out the sample.

(3) From a micropipet, deliver 1000 microliters of each sample beneath the surface of the water in its respective tube, blow out gently and rinse the pipet twice with a complete filling with upper layer water; blow out the residual liquid. Vortex tube for 5 seconds. The micropipets selected must be capable of delivering required volumes within a tolerance of ±2 microliters; a sample dilutor capable of repetitive deliveries with a precision of 0.2 percent (1 C.V.) may be used.

(4) Pipet 10.0 milliliters of TCA precipitant into the tube; cap tightly, shake vigorously for 10 seconds, and place in a rack. Use one transfer pipet for all deliveries of TCA.

(5) Mix each tube by vortexing; then centrifuge. If a smaller tube is required, transfer about 12 milliliters of the mixture to a clean tube for certification. Cap and centrifuge at approximately 1000 x gravity (g) for 10 minutes. Two shorter centrifugations may be necessary to clear the supernate, the first being used to provide clean supernate with which to wash down the sides of the tube above the liquid level. Bring the tubes to room temperature in a temperature-controlled water bath before measuring aliquants of the supernate.

(b) *Color reaction.* (1) A complete set of blanks and standards is run at the beginning and end of each run, using the duplicate dilutions of standards in TCA.

(2) Deliver 2.00 milliliters of each clear supernate into labeled reaction tubes from a 2-milliliter transfer (TD) pipet by letting it drain against the side of the tube; when the level has reached the tip, the pipet is drawn upwards against the glass and removed. A set of pipets must be selected so that repetitive deliveries of water can achieve a precision of 0.2 percent (1 C.V.); if a single pipet is used for all samples, it must be rinsed twice with each succeeding sample before measurement.

(3) To each tube, add 7.0 milliliters of o-toluidine reagent and mix well by vortexing. Delivery may be made from a single transfer (TD) pipet or from a reagent dispenser whose precision of repetitive delivery has been demonstrated to fall within 0.2 percent (1 C.V.).

(4) Rack and cover all tubes, and place the rack in a vigorously boiling water bath for exactly 12 minutes.

(5) Remove the rack from the bath and immerse it in an ice-water bath up to the level of the reagent mixture for 10 minutes (slow rates of cooling may affect the results). Then bring contents to room temperature in a water bath for 10 minutes.

(6) Again mix well by a brief vortexing and perform the photometry by transferring each sample to a rectangular cuvet. If a single cuvet or a flow-through cuvet is used for all readings, it is rinsed twice with portions of about 1 milliliter of the solution to be read; if more than one cuvet is used, results must be corrected for any differences in pathlength. With uniformly rapid cooling, the color is relatively stable, but readings should not be delayed. In a long run, check for fading between the initial and final standard curves.

(7) With the wavelength set to 630 nanometers, zero the photometer with reagent water. Read absorbance of the first set of blank and standards, then specimens and controls, and finally the second set of blank and standards. The zero setting of the photometer is checked with water after each 10 readings. If the absorbance reading drifts beyond ±0.001 absorbance during the measurements, it must be checked more often and the instrument rezeroed.

(8) Refer to paragraph (d) of this section for data evaluation and computations.

(2) *Glucose oxidase method for the determination of glucose in serum, plasma, and cerebrospinal fluid—(i) General.* This procedure is applicable to serum, plasma, and cerebrospinal fluid. It utilizes two oxidoreductases for the enzymatic conversion of glucose. The first, glucose oxidase, oxidizes beta-D-glucose (and ultimately the alpha epimer in solution) to gluconic acid. Hydrogen peroxide is formed, and peroxidase acts on the peroxide and a chromogenic hydrogen donor (orthodianisidine in this method) to yield water and the oxidized form of the chromogen.

Glucose oxidase reaction

$C_6H_{12}O_6$ (Beta-D-glucose) + H_2O (Water) + O_2 (Oxygen (air)) —(glucose oxidase)→ $C_6H_6(OH)_5COOH$ (D-gluconic acid) + H_2O_2 (Hydrogen peroxide)

Peroxidase reaction

H_2O_2 (Hydrogen peroxide) + $[C_6H_3(OCH_3)NH_2]_2$ (Orthodianisidine) —(peroxidase)→ oxidized chromogen (yellow) + $2H_2O$ (Water)

The proteins are removed from the sample by the barium hydroxide-zinc sulfate reagent of Somogyi, and an aliquant of the resultant supernate is taken for the procedure. The zinc hydroxide precipitation also effectively decreases the levels of the other reducing substances which can compete with the chromogen as hydrogen donors. Sulfuric acid is added to stop the reaction and convert the colored product from yellow to rose-pink. The amount of color produced is proportional to the amount of glucose oxidized and is measured in a spectrophotometer at a wavelength of 540 nanometers. The method is basically that of Fales (Fales, F.W., "Standard Methods of Clinical Chemistry," Vol. 4, pp. 101–112, 1963).

(ii) *Reagents and chemical specifications for the glucose oxidase method—(a) Chemical specifications.* (1) Zinc sul-

fate, heptahydrate. ACS specifications (4th ed., p. 642 [2]).

(2) Barium hydroxide, octahydrate. ACS specifications (4th ed., p. 100 [2]).

(3) Sodium phosphate, dibasic, anhydrous. ACS specifications (4th ed., p. 559 [2]).

(4) Potassium phosphate, monobasic, anhydrous, ACS specifications (4th ed., p. 480 [2]).

(5) Glycerol. ACS specifications (4th ed., p. 269 [2]).

(6) Methanol. ACS specifications (4th ed., p. 372 [2]).

(7) Sulfuric acid. ACS specifications (4th ed., p. 597 [2]).

(8) Ortho-dianisidine, reagent grade.

(b) *Enzyme specifications.* Enzymes for the glucose oxidase analysis should meet the following specifications, and should be stored in a desiccator at 4° C.

(1) *Glucose oxidase.* Purified from *Aspergillus niger*. Minimal activity: 13,000 International Units (IU) per gram. One IU is defined as the amount of glucose oxidase required to oxidize 1 micromole of glucose to gluconic acid and H_2O_2 per minute at pH 5.1 at 35.0° C. Catalase, amylase, invertase, maltase and other disaccharidases should be absent.

(2) *Peroxidase.* RZ (Reinheit Zahl) no less than 1.0 from horseradish; salt-free powder. Activity: approximately 100 to 150 purpurogallin units per milligram (one 20-second purpurogallin unit equals the formation of 1 milligram of purpurogallin in 20 seconds from pyrogallol at pH 6.0 at 20.0° C).

(c) *Reagents for protein precipitation—(1) Zinc sulfate solution, 22.0 grams/liter.* Dissolve 22.0 grams of zinc sulfate ($ZnSO_4 \cdot 7H_2O$) in approximately 900 milliliters of freshly prepared, hot CO_2-free distilled water. When the solution has cooled to room temperature, transfer to a 1-liter volumetric flask, dilute to 1000 milliliters with CO_2-free water, mix well, and transfer to a glass reagent bottle. Stopper tightly.

(2) *Barium hydroxide solution, saturated.* Dissolve 80 grams of barium hydroxide ($Ba(OH)_2 \cdot 8H_2O$) from a freshly opened bottle in approximately 950 milliliters of freshly prepared CO_2-free, hot, distilled water. When the solution has cooled to room temperature, transfer to a 1-liter volumetric flask, dilute to 1000 milliliters with CO_2-free water, mix well, and transfer to a storage bottle fitted with a soda-lime trap protected with a short column of silica gel. The life of the soda-lime trap is relatively short, and must be replaced often. Allow the excess barium hydroxide and any carbonates to settle overnight before making the dilution below.

(3) *Barium hydroxide solution, approximately 0.11N.* Without disturbing the precipitate, dilute 245 milliliters of saturated barium hydroxide to 1 liter with CO_2-free water. Titrate 10.0 milliliters of zinc sulfate solution with this dilute barium hydroxide to a faint pink endpoint with phenolphthalein indicator (2 drops of 0.5 percent indicator in 95 percent ethanol). The result should be such that 10.0 milliliters of zinc sulfate solution require 10.0±0.1 milliliter of barium hydroxide solution. If this limit is exceeded, add either saturated hydroxide or CO_2-free water in the appropriate calculated quantities to the barium hydroxide solution and repeat the titration. Transfer the adjusted barium hydroxide solution to a reagent bottle fitted with a soda-lime trap and a syphon or decanting tube system. Each month check equivalence of this solution by titration.

(d) *Reagents for enzymatic and color reactions—(1) Glycerol-buffer solution, pH 7.0.* Dry the necessary quantities of mono- and dibasic phosphates in a 110° C oven. Dissolve 3.48 grams of anhydrous dibasic sodium phosphate (Na_2HPO_4) and 2.12 grams of anhydrous monobasic potassium phosphate (KH_2PO_4) in 600 milliliters of reagent water, then add 400 milliliters of glycerol. Mix well, measure the pH and, if necessary, adjust the pH of the solution to 7.0±0.1. Transfer to a reagent bottle and store at 4° C. To prolong the stability of the reagent, it may be membrane-filtered into a sterile storage bottle. A preparation free of bacteria, stored at 4° C will remain stable for at least 4 months.

(2) *Enzyme solution.* Dissolve 250 milligrams of glucose oxidase in about 10 milliliters of glycerol-buffer solution. In a separate container, dissolve 50 milligrams peroxidase in another 10-milliliter portion of glycerol-buffer. Transfer both enzyme solutions to a 1-liter volumetric flask by washing it with the glycerol-buffer solution and bring to volume with the glycerol-buffer solution. Mix well, divide into 10 separate 100-milliliter portions and store these in brown screw-cap bottles. Store at −20° C. The reagent is stable for about 3 months.

(3) *Ortho-dianisidine-methanol solution.* Dissolve 200.0 milligrams of ortho-dianisidine in 20 milliliters of absolute methanol. Mix well and store in a tightly capped brown plastic bottle at 4° C. The solution is stable for about 2 months.

(4) *Glucose oxidase reagent.* Bring to room temperature one 100-milliliter bottle of enzyme solution and a small portion of ortho-dianisidine-methanol solution. Pipet 1.0 milliliters of ortho-dianisidine-methanol solution into a flask. Quickly pour the enzyme solution into the flask with swirling (to prevent precipitation of the dianisidine) and mix well. Use within 1 day.

(5) *Sulfuric acid (H_2SO_4), 8N.* Chill 700 milliliters of reagent water in an Erlenmeyer flask in an ice bath. Add slowly, and with rapid stirring, 200 milliliters of sulfuric acid. Mix thoroughly and store in a glass-stoppered reagent bottle.

(iii) *Procedure for glucose oxidase analysis of glucose—(a) Preparation of deproteinized supernates ("Somogyi filtrates").* (1) Pour sufficient portions of each standard into clean cappable tubes, and return standard to refrigerator. Bring all specimens, standards and reagent water to room temperature by placing in a temperature-controlled water bath. Label a sufficient number of 18 x 150 millimeter cappable tubes to accomodate:

Types of sample:	*Preparation*
Blank (zero concentration standard).	Prepared with benzoic acid diluent as a sample and carried through the procedure for photometric blanking.
Working standards.	50, 100, 150, 200, and 250 mg/dl (2.78, 5.55, 8.33, 11.10 and 13.88 mM/l, respectively) standards; if specimens up to 300 mg/dl (16.65 mM/l) are to be analyzed, include a 300 mg/dl working standard.
Two biologic control samples.	Of appropriate concentration and analytical history.
Specimens -----	Specimens above 300 mg/dl (16.65 mM/l) require dilution with water.

(2) Deliver 10.0 milliliters of 0.11*N* barium hydroxide solution into each tube from the same volumetric transfer (TD) pipet.

(3) From a micropipet, deliver 1000 microliters of the first sample beneath the surface of the solution into its labeled tube, blow out gently, and rinse the pipet completely twice with upper layer solution. Vortex the tube for 5 seconds.

(4) Pipet 10.0 milliliters of zinc sulfate solution into the tube. Cap and shake vigorously (or vortex for 5 seconds), set aside and proceed to the next sample. Proceed similarly with all samples. This procedure may be conducted by a reliable sample dilutor meeting the precision requirements previously specified, and with the same precautions about cross-contamination between samples and reagents. A fixed volume dispenser of high reliability and precision may be used to dispense the zinc sulfate solution. If suitable precision is demonstrated in these semiautomated steps, all of the above volumes may be scaled to half.

(5) After again mixing each tube by a short vortexing, centrifuge (or if necessary, transfer about 12 milliliters of the mixture to a clean tube). Cap and centrifuge at approximately 1000 x gravity (g) for 10 minutes. Two shorter centrifugations may be necessary to clear the supernatant solution, the first being used to wash down the sides of the tube above the liquid level. Bring the tubes to room temperature in a temperature-controlled water bath before measuring the aliquants of the supernate.

(b) *Enzymatic and color reactions.* (1) Label clean screw-cap 15 x 125 millimeter tubes for an initial set of blank and standards; unknown and control specimens; and a final set of blank and standards.

(2) From a transfer pipet deliver 2.00 milliliters of glucose oxidase reagent and bring to 37° ±0.5° C in a water bath.

(3) From a micropipet deliver 500 microliters of each sample supernate into its proper tube, rinsing twice by a complete filling with the reagent; blow out the residual liquid each time. The tube is

[2] See footnote 2 at p. 24142, *supra*.

replaced in the 37° C water bath for exactly 30 minutes. The addition of each sample should be at timed intervals, e.g., 30 seconds. This procedure requires selection of a set of calibrated pipets that are verified to contain volumes that match within a tolerance of 1 microliter (0.2%). A sample dilutor with a demonstrated capability for this tolerance may be used for the steps set forth in paragraph (e) (2) (iii) (b) (2) and (3) of this section.

(4) At the end of its 30-minute incubation period, remove each tube and immediately pipet 4.00 milliliters of 8*N* sulfuric acid into the tube. Cap, mix vigorously, and place the tube in a water bath at room temperature. The color is stable for an hour after the addition of sulfuric acid, but the samples are read in their original sequence to assure a uniform reading time.

(5) For photometry, a single rectangular sample cuvet or flow-through cuvet is recommended. Rinse twice between each sample with approximately 1 milliliter volumes.

(6) With the wavelength set to 540 nanometers, zero the photometer with reagent water. Read absorbance of initial set of blank and standards, then the specimens and controls, and finally the second set of blank and standards. The zero setting should be checked periodically with water to ascertain stability. If after setting to zero the absorbance reading has drifted beyond ±0.001 absorbance, the instrument must be rezeroed with a fresh water sample. Drift beyond ±0.002 absorbance from the initial setting represents unacceptable photometer performance.

(7) Refer to paragraph (d) of this section for data evaluation and computations.

(3) *Hexokinase/glucose-6-phosphate dehydrogenase (G6PDH) method for the determination of glucose in serum, plasma, or urine.*

(i) This is a reaction completion method based upon the following reactions:

HEXOKINASE REACTION

$$\text{D-glucose} + \text{ATP} \xrightarrow[Mg^{++}]{\text{(yeast hexokinase)}} \text{D-glucose-6-phosphate} + \text{ADP}$$

Where:
ATP = Adenoscine triphosphate.
ADP = Adenoscine diphosphate.

G6PDH REACTION

$$\text{D-glucose-6-phosphate} + \text{NAD} + \xrightarrow[(L.\ mesenteroides)]{\text{G6PDH}} \text{6-phosphoglucono-}\Delta\text{-lactone} + \text{NADH}$$

Where:
NAD = Nicotinamide-adenine dinucleotide.
NADH = Nicotinamide-adenine dinucleotide, reduced.

Due to rapid hydrolysis of the gluconolactone, the reaction sequence goes essentially to completion. Utilizing this coupled reaction, one mole of NAD is reduced for each mole of glucose phosphorylated. The gain in absorbance at 340 nanometers by the NADH produced is used to quantify the assay, which is linear to a glucose concentration of 600/mg/dl (33.3 mM/l under the conditions of the analysis.

(ii) Although other hexoses (most importantly fructose and mannose) are phosphorylated by hexokinase, they are normally present in serum or plasma in negligible amounts in comparison with glucose. These other hexose phosphates must be isomerized before entering into the second reaction; if the enzyme preparations can be kept free of contamination with isomerases, the reaction is highly specific.

(iii) The above reaction sequence, or the analogous coupled reaction using yeast dehydrogenase and nicotinamide-adenine dinucleotide phosphate (NADP), is frequently applied directly to samples in a useful clinical method; however, for the most reliable results, deproteinization is required. This procedure utilizes a Somogyi (barium-zinc hydroxide) deproteinized supernate to which the enzymatic reactions are applied.

(iv) Work on the development of the following method was undertaken by the Center for Disease Control in collaboration with the Subcommittee for a Glucose Referee Method of the American Association of Clinical Chemists and is being prepared for joint publication. Coupling of these reactions was first employed by Slein et al., in an assay for hexokinase (Slein, M. W., G. T. Cori, and C. F. Cori, *Journal of Biological Chemistry*, 186:763, 1950).

(v) Reagents for the hexokinase/G6PDH method—(*a*) *Chemical specifications.* (*1*) Zinc sulfate, heptahydrate. ACS specifications (4th ed., p. 642 [3]).

(2) Barium hydroxide, octahydrate. ACS specifications (4th ed., p. 100 [3]).

(3) Magnesium acetate. ACS specifications 4th ed., p. 329 [3]).

(*4*) Tris(hydroxymethyl) aminomethane (Tris), reagent grade.

(5) Tris hydrochloride. Reagent grade. grade.

(*6*) Nicotinamide adenine dinucleotide, oxidized (NAD). Purity of 98 percent or better.

(7) Adenosine triphosphate (ATP). Purity of 98 percent or better.

(*b*) *Enzyme specifications—(1) Hexokinase.* The content of contaminants should not exceed the following limits:

	Percent
Glucose-6-phosphate dehydrogenase	0.005
Glutathione reductase	.005
Phosphoglucose isomerase	.003
Myokinase	.001
6-phosphogluconate dehydrogenase	.001

One international unit (IU) of hexokinase activity is defined as that amount of enzyme which will catalyze the conversion of one micromole of glucose per minute at 25° C. The activity may be determined by the method of: (Darrow R.A., and S.P. Colwick, "Methods in Enzymology," *V*:226, 1962).

(2) *Glucose-6-phosphate dehydrogenase (G6PDH) (D-glucose-6-phosphate NAD oxidoreductase).* The source of the enzyme is *Leuconostoc mesenteroides*, available as suspension in ammonium sulfate solution or as lyopholized powder. One IU of G6PDH is that amount of enzyme which reduces one micromole of NAD per minute at 25° C. The activity may be determined by the method of: (Olive C., and H.R. Levy, *Biochemistry*, 6:730, 1971).

(3) *Reagents for protein precipitation.*

ZINC SULFATE SOLUTION, 22.0 GRAMS/LITER

Dissolve 22.0 grams of zinc sulfate ($ZnSO_4 \cdot 7H_2O$) in approximately 900 milliliters of freshly prepared, CO_2-free, hot distilled water. When the solution has cooled to room temperature, transfer to a 1-liter volumetric flask, dilute to 1000 milliliters with CO_2-free water, mix well, and transfer to a glass reagent bottle. Stopper tightly.

BARIUM HYDROXIDE SOLUTION, SATURATED

Dissolve 80 grams of barium hydroxide ($Ba(OH)_2 \cdot 8H_2O$) from a freshly opened bottle in approximately 950 milliliters of freshly prepared CO_2-free, hot distilled water. When the solution has cooled to room temperature, transfer to a 1-liter volumetric flask, dilute to 1000 milliliters with CO_2-free water, mix well, and transfer to a storage bottle fitted with a soda-lime trap protected with a short column of silica gel. The life of the trap is relatively short, and must be replaced often. Allow the excess barium hydroxide and any carbonates to settle overnight before making the dilution below.

BARIUM HYDROXIDE SOLUTION, APPROXIMATELY 0.11 *N*

Without disturbing the precipitate, transfer 245 milliliters of saturated barium hydroxide to a 1-liter volumetric flask. Dilute to 1000 milliliters with CO_2-free water. Titrate 10.0 milliliters of zinc sulfate solution with this dilute barium hydroxide solution to a faint pink endpoint with phenol-phthalein indicator (2 drops of 0.5 percent indicator in 95 percent ethanol). The result should be such that 10.0 milliliters of zinc sulfate solution require 10.0±0.1 milliliters of barium hydroxide solution. If this limit is exceeded, add add either saturated hydroxide or CO_2-free water in the appropriate calculated quantities to the barium hydroxide solution and repeat the titartion. Transfer the adjusted barium hydroxide solution to a reagent bottle fitted with a soda-lime trap and a syphon or decanting tube system. Each month check equivalence of this solution by titration.

(*4*) *Tris buffer, 0.1M, pH 7.5 at 25° C, containing 4 mM magnesium acetate.*

TRIS·HCL STOCK SOLUTION

31.52 grams Tris·hydrochloride, dissolved and diluted to 2000 milliliters with reagent water in a 2-liter volumetric flask.

TRIS BASE STOCK SOLUTION

6.06 grams of Tris base, dissolved and diluted to 500 milliliters with reagent water in a 500-milliliter volumetric flask. These stock solutions, if carefully prepared and protected from mold contamination, can be stored at 4° C for several months.

TRIS-MAGNESIUM BUFFER, PH 7.5

Mix 800 milliliters of the Tris-HCl solution and 200 milliliters of the Tris base solution in a large beaker, and then dissolve 1.1 grams of magnesium acetate in the solution.

[3] See footnote 2, p. 24142, *supra*.

The pH of the mixture is determined at 25° C. If necessary, adjust with either Tris-HCl or Tris base solution to pH 7.5±0.1. Filter the solution through a sterile 0.45 micron membrane filter into a sterilized borosilicate glass screw-cap storage bottle. If carefully handled, this solution may be stored at 4° C for up to 6 months.

(5) *Enzyme reagent.* Dissolve 0.7962 grams of NAD, 0.6614 grams of ATP and 100 IU each of hexokinase and glucose-6-phosphate dehydrogenase in approximately 500 milliliters of Tris-magnesium buffer in a 1-liter volumetric flask. The quantities of these enzymes to be used must be determined by previous assays for activity of the source preparations. Five IU of each enzyme are employed per test (in each 5 milliliters of enzyme reagent). Dilute to volume with Tris-magnesium buffer and mix thoroughly by gentle inversion. Dispense 100-milliliter volumes (100 milliliters yields 19 tests) into clean, dry, screw-cap 125-milliliter heavy borosilicate glass bottles and store at −20° C until used. The enzyme solutions prepared and stored in this manner are stable approximately 6 months. On the day of the assay, remove the enzyme reagent from the freezer and place in a 25° C water bath for about one hour or until equilibrated to 25°±1° C.

(c) *Tests of reagent adequacy.* (1) Set the photometer to zero absorbance with water as a blank. Read the absorbance of the enzyme (5 milliliter enzyme reagent plus 1000 microliters water as sample). The reading should be less than 0.100 absorbance.

(2) The following test is applied for verifying acceptable enzyme activity and for monitoring reagent stability over time: A 600 mg/dl (33.3 mM/1) aqueous glucose standard (30.0 milliliters of stock 10 grams/liter glucose standard diluted with benzoic acid diluent to 50 milliliters in a volumetric flask) is processed by the complete procedure. The resultant absorptivity should lie between 1.6 and 1.8 absorbance, and the absorbance/concentration slope at 600 mg/dl should be less than 95 percent of the average slope obtained when using standards in the 0 to 250 mg/dl range. This reaction should be complete within 6 minutes at 25° C.

(d) *Preparation of deproteinized supernates ("Somogyi filtrates").*

(1) Pour sufficient portions of each standard into clean cappable tubes, and return standards to refrigerator. Bring all specimens, standards, and reagent water to room temperature by placing in a temperature controlled water bath. Label a sufficient number of 18 x 150 millimeter cappable tubes to accomodate:

Type of sample:	*Preparation*
Blank (zero concentration standard).	Prepared with benzoic acid diluent as a sample, and carried through the procedure for photometric blanking.
Working standards.	50, 100, 150, 200, and 250 mg/dl standards (2.78, 5.55, 8.33, 11.10 and 13.88 mM/1, respectively). If specimens up to 300 mg/dl (16.65 mM/1) are to be analyzed, include a 300 mg/dl working standard.
Two biologic control samples.	Of appropriate concentration and analytical history.
Specimens -----	Specimens above 300 mg/dl (16.65 mM/1) require dilution with water.

(2) Deliver 10.0 milliliters of 0.11*N* barium hydroxide solution into each tube from the same volumetric transfer (TD) pipet.

(3) From a micropipet, deliver 1000 microliters of the first sample beneath the surface of the solution into its labeled tube, blow out gently, and rinse the pipet completely twice with upper layer solution. Vortex the tube for 5 seconds.

(4) Pipet 10.0 milliliters of zinc sulfate solution into the tube. Cap and shake vigorously (or vortex for 5 seconds), set aside and proceed to the next sample. Proceed similarly with all samples. A reliable sample dilutor meeting the precision requirements previously specified may be used for the above procedure, with the same precautions about cross-contamination between samples and reagents. A fixed volume dispenser of high reliability and precision may be used to dispense the zinc sulfate solution. If suitable precision is demonstrated in these semi-automated steps, all of the above volumes may be scaled to half.

(5) Again mix each tube by a short vortexing. If necessary, transfer about 12 milliliters of the mixture to a clean tube, cap and centrifuge at approximately 1000 x grams for 10 minutes. Two shorter centrifugations may be necessary to clear the supernatant solution, the first being used to wash down the sides of the tube above the liquid level. Bring the tubes to room temperature in a temperature controlled water bath before measuring the aliquants of the supernate.

(e) *Enzyme reaction.* (1) Label a set of clean screw-cap 15 x 125 millimeter tubes for an initial set of blank and five standard "supernates"; supernate of each specimen and control; a final set of blank and five standard "supernates."

(2) To each tube, add 5.0 milliliters of enzyme reagent from a single transfer (TD) pipet or from a reagent dispenser with a demonstrated precision of repetitive delivery of 0.2 percent (1 C.V.). Place the tubes in a water bath of 25° ±1° C.

(3) From a micropipet, deliver 1000 microliters of each clear supernate beneath the surface of the enzyme reagent in its proper tube, blow out gently, and rinse the pipet twice with a complete filling with upper layer liquid; blow out the residual liquid. Cap the tube, mix by gentle inversion, five times, and place in the water bath for 10 minutes at 25° ±1° C. Automatic pipetting or diluting devices capable of 0.2 percent precision in repetitive delivery may be employed for steps set forth in paragraph (e) (3) (v) (e) (2) and (3) of this section, using the 5.0 milliliter enzyme reagent as the fluid to flush out 1000 microliters of supernate.

(4) After 10 minutes of incubation, again mix well by slow inversion and perform the photometry within 30 minutes for all samples. Timing is not critical but photometry is performed in the serial order of addition, so that each sample is read at approximately the same time interval from the addition of sample. Transfer each sample to a 10.0 millimeter cuvet. If one cuvet or a flow-through cuvet is used, it is rinsed with several 1-milliliter portions of the solution to be measured; if matched cuvets are used, they must be corrected for any differences in path-length.

(5) With the wavelength set at 340 nanometers, zero the photometer with reagent water. Read absorbance of the initial set of blank and standards. Recheck zero with reagent water after a complete rinsing of the cuvet. Read specimens and controls. Recheck zero with reagent water and read the final set of blank and standards. If absorbance reading drifts beyond ±0.001 absorbance during the measurements, it must be checked more often and the instrument rezeroed with reagent water.

Interested persons may, on or before October 28, 1974, file with the Hearing Clerk, Food and Drug Administration, Rm. 6–86, 5600 Fishers Lane, Rockville, MD 20852, written comments (preferably in quintuplicate) regarding this proposal. Comments may be accompanied by a memorandum or brief in support thereof. Received comments may be seen in the above office during working hours, Monday through Friday.

Dated: June 24, 1974.

A. M. SCHMIDT,
Commissioner of Food and Drugs.

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